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Cryopreserved neonatal hepatocytes may be a source for transplantation; – evaluation of functionality towards clinical use

Charlotte A. Lee¹, Anil Dhawan^{2*}, Valeria Iansante¹, Sharon Lehec¹, Shirin Elizabeth Khorsandi³, Celine Filippi, Simon Walker¹, Raquel Fernandez-Dacosta¹, Nigel Heaton³, Sanjay Bansal², Ragai R Mitry^{1*}, Emer Fitzpatrick^{2*}.

¹Dhawan Lab, Institute of Liver Studies, King's College London, Faculty of Life Sciences and Medicine, King's College London at King's College Hospital, London UK, ² Paediatric Liver, GI and Nutrition Centre, Mowat Labs, King's College London, Faculty of Life Sciences and Medicine, King's College London at King's College Hospital, London UK³ Institute of Liver Studies, King's College Hospital NHS foundation Trust, London UK

*Joint senior authors

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Abbreviations: adult hepatocyte (AH), acute liver failure (ALF), bovine serum albumin (BSA), donation following neurological determination of death (DNDD), donation after circulatory determination of death (DCDD), hepatocyte transplantation (HT), National Health Service Blood and Transplant (NHSBT), neonatal hepatocyte (NH), liver transplantation (LT), phosphate buffered saline (PBS), polyvinyl chloride (PVC), portal vein (PV), reverse-transcriptase polymerase chain reaction (RT-PCR), Royal College of Paediatrics and Child Health (RCPCH), sulforhodamine B (SRB), university of Wisconsin solution (UW), Williams E (WE)

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Corresponding Address:

Dr Emer Fitzpatrick
Pediatric Liver, GI and Nutrition Centre
King's College Hospital NHS Foundation Trust
King's College Hospital
Denmark Hill
London SE5 9RS

020 3299 4375
emer.fitzpatrick@kcl.ac.uk

Dr Emer Fitzpatrick MD FRCPCH MBBS

Abstract

Neonatal livers are a potential source of good quality hepatocytes for clinical transplantation. We compared viability and function of neonatal and adult hepatocytes, and report their clinical use both intra-portal and in alginate microbeads. Following isolation from donor livers, hepatocyte function was assessed using albumin, alpha-1 antitrypsin and factor VII. Metabolic function was investigated by measuring resorufin conjugation, ammonia metabolism, UGT enzyme activity and cytochrome P450 function following induction. Activation of the instant blood mediated inflammatory (IBMIR) reaction by neonatal and adult hepatocytes was investigated using an *in vitro* blood perfusion model and tissue factor expression was analysed using qPCR. Clinical hepatocyte transplantation was undertaken using standard protocols. Hepatocytes were isolated from 14 neonatal livers, with an average viability of $89.4 \pm 1.8\%$ (Mean \pm SEM) and average yield of $9.3 \times 10^6 \pm 2.0 \times 10^6$ cells/g. Hepatocytes were isolated from 14 adult livers with an average viability of $78.0 \pm 2.4\%$ and yield $2.2 \times 10^6 \pm 0.5 \times 10^5$ cells/g. Neonatal hepatocytes had significantly higher viability post cryopreservation than adult hepatocytes, with better attachment efficiency and less plasma membrane leakage. There were no differences in albumin, alpha-1 antitrypsin and factor VII synthesis between neonatal and adult hepatocytes ($P > 0.05$). Neonatal cells had inducible phase I enzymes as assessed by cytochrome p450 function and functional phase II enzymes, in which activity was comparable to adult hepatocytes. In an *in vitro* blood perfusion model adult hepatocytes elicited increased thrombus formation with a greater consumption of platelets and white cells compared with neonatal hepatocytes (28.3×10^9 vs 118.7×10^9 and 3.34×10^9 vs 6.63×10^9 , $P < 0.01$). Intraportal transplantation and intraperitoneal transplantation of alginate encapsulated hepatocytes was safe and preliminary data suggest the cells may activate the immune response to a lesser degree than adult cells. **Conclusion:** We have shown neonatal hepatocytes have excellent cell viability, function and drug metabolism making them a suitable alternative source for clinical hepatocyte transplantation.

Introduction

Hepatocyte transplantation (HT) has demonstrated some promise as an alternative to liver transplantation (LT) for children with liver-based metabolic diseases and acute liver failure (ALF). Due to the shortage of donor organs, pediatric patients often face a long-wait for an appropriately sized organ of sufficient quality to become available. As a result, hepatocyte transplantation is being explored as an alternative. Significant improvement in metabolic function has been demonstrated following hepatocyte transplantation in children with liver-based metabolic disease, however efficacy beyond 18 months has yet to be shown.

Human hepatocytes are isolated from whole donor livers or segments unsuitable for whole organ transplantation, using a collagenase perfusion technique and purified using centrifugation (1–3). For patients with liver-based metabolic diseases, the cells are administered under radiological guidance into the portal vein, from which they migrate across the sinusoidal endothelial barrier and engraft in the parenchyma. Successful engraftment depends on the ability of hepatocytes to survive the innate immune system and successfully transverse the sinusoids. Viability and function of transplanted hepatocytes is associated with the quality of liver tissue from which they are isolated. In the case of children with ALF, administration of hepatocytes intra-portally is challenging, particularly in the coagulopathic, critically ill patient. As an alternative, microbeads of alginate encapsulated hepatocytes which are injected into the intraperitoneal cavity is a newly described approach, in which the semi-permeable gel alginate allows the entry of oxygen and nutrients into the microbead while simultaneously protecting the hepatocytes against the immune system (4). One of the major limitations of the technique is that the cells are largely derived from poor quality livers that have otherwise been rejected for transplantation, thus the quality of the cells themselves is often very poor.

Neonatal livers have recently been explored as a cell source for hepatocyte transplantation to overcome this major limitation. Neonatal livers are rarely used for solid organ transplantation due to the high incidence of hepatic artery thrombosis, which may result from decreased or absent protein C

synthesis by the donor liver (5). Instead they may be offered for hepatocyte isolation for the purpose of cell transplantation. Transplantation of human neonatal hepatocytes into a rat model of Crigler Najjar syndrome showed animals that received neonatal hepatocytes had significantly higher concentrations of UGT1A1 and conjugated bilirubin expression at both 3 days and 6 months compared to adult cells. Functionality of neonatal hepatocytes has also been shown in 4 patients with severe urea cycle disorders, showing metabolic stabilization for 4-13 months following transplantation (6,7).

In the UK, the introduction of new guidelines defining brain death in newborns has increased the number of donor organs that are offered for hepatocyte isolation, resulting in an increased requirement to characterize these cells in the context of hepatocyte transplantation (8). The aim of this study was to investigate whether neonatal hepatocytes are a suitable alternative to adult hepatocytes. To investigate this we compared viability, functionality, drug metabolism and pro-coagulant effects of these cells. We also describe three cases in which hepatocyte transplantation was undertaken using neonatal cells.

Materials and Methods

Ethical approval for the isolation of primary human hepatocytes was obtained from the National Research Ethics Service (King's College Hospital LREC (LREC 01-016)). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Organs are donated through the National Health Service Blood and Transplant (NHSBT) and offered for hepatocyte transplantation following decline for solid organ transplantation with appropriate consent in place for clinical and research use.

Neonatal Liver Procurement

For donation after circulatory determination of death (DCDD), maximum warm ischemic time (WIT) was usually set at 90 minutes. In the absence of an alternative definition for neonatal WIT, the adult definition is used, which is the time from oxygen saturation of 70% or systolic pressure of 50mmHg (depending on which agonal observation occurs first), to aortic cannulation. DCDD and donation following neurological determination of death (DNDD) were accepted according to guidelines published by the RCPCH (8). The cold ischaemia time is defined as the time from aortic cannulation to hepatocyte isolation. For paediatric DCDD procurement, asystole was awaited after withdrawal of life support. Following declaration of death and a 5 min stand off period, a thoraco-abdominal incision was made, followed by abdominal aorta cannulation and cross clamp in the chest. Depending on vessel size, the portal vein (PV) was cannulated either in the hilum or via the small mesenteric vein in the root of the small bowel mesentery. In situ 500ml ice-cold University of Wisconsin Solution (UW) was perfused through the aorta and PV followed by rapid hepatectomy. A further 250-500ml of UW was perfused through the PV and hepatic artery until the effluent became clear. Cold static storage was the standard.

Neonatal Hepatocyte Isolation

Primary hepatocytes were isolated from whole livers as described by Mitry et al (9,10). Briefly, major blood vessels on the liver surface were cut, cannulated, and secured through suturing. The tissue was

perfused at a flow rate of 50ml/min with buffer (calcium-free HBSS, 1M HEPES and 0.5mM EGTA), followed by calcium-free HBSS and EMEM containing 0.05% collagenase (*VitaCyte, Indianapolis, USA*). Once digested, the tissue was minced and sieved. Hepatocytes were purified by washing three times in ice-cold EMEM and centrifuged at 50xg at 4°C for 5 minutes. Cells used were cryopreserved in UW solution with 5% glucose and 10% DMSO using a controlled rate freezer (*Kryo 10, series III, Planer Products, Ltd, Middlesex, UK*) and stored at -140°C.

Thawing and culture of cryopreserved primary hepatocytes

All assays were carried out using hepatocytes that had been cryopreserved and thawed. Cryopreserved hepatocytes were thawed at 37°C, re-suspended in Williams E (WE) medium and centrifuged at 50xg for 5 minutes at 4°C. Cells were re-suspended and viability determined using the trypan blue exclusion assay. Hepatocytes were cultured in WE containing; 50µg/ml heat-inactivated foetal bovine serum, 10mM HEPES, Penicillin (100U/ml)- Streptomycin (100µg/ml), Insulin(10mg/L)– Transferrin(5.5mg/L)-Selenium(6.7µg/L), 10⁻⁷M dexamethasone and 2mM L-glutamine. 96 well plates were coated with 0.1mg/ml rat-tail collagen type 1 overnight and washed with PBS.

Assessment of hepatocyte viability, attachment and plasma membrane damage

MTT assay

Hepatocyte viability was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were cultured for 24h at 37°C, 5% CO₂, supernatant removed and cultured with serum-free medium containing 0.5mg/ml of MTT (*Sigma Aldrich, Dorset, UK*) for 4 hours. The produced formazan was dissolved in DMSO and the optical density read at 570nm on a Dynex MRX microplate reader (see supplementary information for more details).

Sulphorhodamine B (SRB) assay

A Sulphorhodamine B (SRB) assay was carried out to determine cell attachment. Hepatocytes were cultured for 24h at 37°C, 5% CO₂. Supernatant was removed and replaced with 50% Trichloroacetic

acid (TCA), cells were washed and SRB added before solubilisation in 1% acetic acid. The optical density was read at 564nm on a Dynex MRX microplate reader (see supplementary information for more details).

Lactate dehydrogenase assay

Plasma membrane damage following cryopreservation was measured using a lactate dehydrogenase (LDH) assay (*Pierce LDH cytotoxicity kit, Thermo Fisher Scientific, Paisley, UK.*) Cells were cultured for 24h at 37°C, 5% CO₂. Sterile water was added to control cells, which represented spontaneous LDH activity. Lysis buffer was added to other wells, which represented maximum LDH activity. Reaction mixture was added and absorbance read at 490nm and 680nm (background signal). LDH activity was quantified as the percentage of spontaneous LDH activity over the maximum LDH activity (see supplementary information for more details).

Immunofluorescent Staining

For visualization of albumin expression cells were fixed with 4% PFA, permeabilised with 0.01% Triton X-100, blocked with PBS + 3% BSA and incubated with goat anti-human albumin antibody 1:100 (*Bethyl Laboratories, Inc, Texas, USA*), followed by a goat anti-mouse 594 secondary antibody 1:1000 (*Invitrogen*). For analysis of foetal markers, a mouse monoclonal EpCAM antibody 1:50 (*Santa Cruz, Heidelberg, Germany*) and a mouse monoclonal alpha-1 fetoprotein (AFP), followed by a goat anti-mouse 488 secondary antibody 1:200 (*Thermo Fisher Scientific, Paisley, UK*) were used. Images were taken on an Inverted Microscope Leica DMi8 x20 magnification.

Assessment of hepatocyte metabolic function

Albumin, Alpha-1 Antitrypsin and Factor VII synthesis

For functional assays, media was collected 12 hours post-plating and enzyme immunoassays carried out for human Albumin (*Bethyl Laboratories, Inc, Texas, USA*), Alpha-1 Anti-trypsin (*Bethyl Laboratories, Inc, Texas, USA*) and Factor VII (*Assay Pro, Missouri, USA*) according to the manufactures instructions (see supplementary information for more details). Values were normalized

to protein content using a Bicinchoninic Acid Assay (BCA) Protein Assay (see supplementary information).

Ammonia Metabolism

Urea synthesis was measured using a QuantiChrom™ Urea Assay kit (*Universal Biologicals, Cambridge, UK*). Cells were washed with PBS and treated with 5mM ammonium chloride (*Sigma Aldrich, Dorset, UK*) for 6 hours before measurement of urea synthesis (see supplementary information for more details).

Phase II assay

Phase II activity was measured by quantifying the conjugation of resorufin. Cells were cultured for 24h at 37°C, 5% CO₂. 10µg/ml of resorufin was added for 2 hours at 37°C, 5% CO₂. Resorufin values were measured at 0hrs and 2hrs and the concentration of resorufin calculated using a standard curve (100ng/ml-1.56ng/ml). Values at 2hrs were subtracted from the 0hr time point to measure decrease of fluorescent resorufin due to its conjugation.

UDP glucuronosyltransferase activity (UGT) activity

UGT activity was measured using a UGT-Glo assay (*Promega, Southampton, UK*). Cells were cultured at 37°C, 5% CO₂ for 24hrs. To measure UGT activity, cells were treated with or without 50µM uridine 5'diphosphoglucuronic acid (UDPGA) and proluciferin substrates (50µM UGT multienzyme mix) for 1 hour at 37°C, 5% CO₂). Following incubation, 50µl of detection agent was added to samples and the luminescence recorded immediately on FLUOstar Omega Microplate Reader (*BMG Labtech, Ortenberg, Germany*) from 0 to 20 minutes every 1 minute, integration time 1 second. Values shown are the cells without UDPGA minus the cells with UDPGA as a percentage of the cells without UDPGA.

7-Ethoxyresorufin-O-Deethylase (EROD) assay

Cytochrome P450-1A1/1A2 activity was assessed by measuring the conversion of 7-ethoxyresorufin to resorufin. Cells were cultured for 24h at 37°C, 5% CO₂. Cells were treated with 20µM 7-

ethoxyresorufin and 1.5mM salicylamide and the concentration of resorufin calculated using a standard curve (100ng/ml-1.56ng/ml).

Cytochrome P450 assays

Cytochrome P450-1A1, 1A2, 2C9, 3A4 and 3A7 activity were measured using a commercial P450-Glo™ cell-based assay (*Promega, Southampton, UK*) according to manufactures instructions. Hepatocytes were cultured for 24h at 37°C, 5% CO₂ and cytochrome p450 activity induced every day for 3 days before detection of cytochrome activity. CYP1A1/1A2 activity was induced using 50μM omeprazole (OMP), CYP2C9 and CYP2B6 activity was induced using 1mM phenobarbital (PHE) and CYP3A4 and CYP3A7 activity was induced using 25μM rifampicin (RIF). Following 3 days of induction, 50μl of luciferin substrate added/well (Luciferin-CEE/CYP1A1 1:50, Luciferin-ME/CYP1A2 1:50, Luciferin-H/CYP2C9 1:50, Luciferin-IPA/CYP3A4 1:1000, Luciferin-CEE/CYP3A7 1:50). Cells were incubated with the luciferin substrate for 60 minutes (CYP3A4) and 3 hours (CYP1A1, 1A2, 3A4 and 3A7). Following incubation, 50μl of detection agent was added to samples and the luminescence recorded immediately on FLUOstar Omega Microplate Reader (*BMG Labtech, Ortenbery, Germany*) from 0 to 20 minutes every 1 minute, integration time 1 second. Values were normalised to DNA content using Quant-IT Picogreen® (*Thermo Fisher Scientific, Paisley, UK*) (see supplementary information for more details).

Chandler loop model

A Chandler loop model was designed based on previous reports (11,12). Briefly, custom-made clinical grade polyvinyl chloride (PVC) tubing (3/16"x1x16"x40cm) coated in heparin using end point attached heparin technology (*Medtronic Carmeda® Bioactive surface, Watford, UK*) was filled with 6mls ABO matched blood containing the relevant samples and closed into circuits with heparin coated 3/16" polystyrene connectors. Tubing loops were rotated at 24rpm and incubated at 37°C for 0, 15, 30 and 60 minutes. Samples were taken with a Pasteur pipette into 1ml EDTA tubes (1.8mg EDTA per milliliter of blood).

Whole blood samples were immediately analysed for full blood counts on an ADIVA 2120 haematology system (*Siemens Healthcare Diagnostics, Surrey, UK*). The remaining samples were centrifuged at 2000xg for 15 minutes to obtain the plasma. Samples were stored at -80°C until analysis.

Cytokine expression was analysed using a high sensitivity Randox HS X Biochip Array designed to measure 12 cytokines/chemokines (IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN- γ , TNF- α , IL-1a, IL-1b, MCP-1 (*Randox Laboratories Ltd, County Antrim, UK*)).

Tissue Factor Expression

mRNA

RNA was extracted from neonatal and adult cells using a miRNeasy kit (*Qiagen, Manchester, UK*). qPCR for TF and β -actin was carried using Taqman® probes (F3 gene, Assay ID Hs1076029_m1, Lot #P150911-007 B12 and β -actin, Assay ID Hs9999903_m1 Lot # P150213_011 A09, *Thermo Fisher Scientific, Paisley, UK*). RNA was converted to cDNA the Omniscript Reverse Transcriptase kit (*Qiagen, Manchester, UK*). The PCR program was 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds and 57°C for 30 seconds, and was run on the Applied Biosystems QuantStudio 7 Flex Real Time PCR system (*Thermo Fisher Scientific, Paisley, UK*). Results were normalised to β -actin and the comparative C_T method used to analyse the results.

Protein expression

For analysis of tissue factor expression, hepatocytes were plated in 4 well chamber slides at 250,000 cells/chamber and incubated at 37°C, 5% CO₂ overnight. Slides were prepared for staining as described in Materials and Methods (2.8.1). Sections were stained with a primary rabbit monoclonal tissue factor antibody (*Abcam, Cambridge, UK*) at a 1:50 dilution for 1 hour at RT, followed by a donkey anti-rabbit ALEXA-Fluor 488 secondary antibody (*Thermo Fisher Scientific, Paisley, UK*) at a 1:200 dilution.

Clinical Hepatocyte Transplantation

A national hepatocyte transplantation programme is in operation at King's College Hospital, London. Children are referred for either intraportal infusion of hepatocytes for liver-based metabolic disease or for intraperitoneal infusion of alginate encapsulated hepatocytes for ALF. Hepatocytes may be used either fresh, or cryopreserved. Hepatocytes must meet minimum viability and microbiological criteria prior to clinical cell transplantation.

For liver-based metabolic disease, radiologically guided intraportal infusion of hepatocytes is undertaken via the umbilical vein if patent and the ductus venosus is closed or accessed surgically via the inferior mesenteric vein at laparotomy. Cells are infused while measuring portal pressure. Standard immunosuppression is with corticosteroids, basiliximab and tacrolimus. Prophylactic broad spectrum antibiotics are used for 48 hours.

Children with ALF who meet criteria for LT may be candidates for hepatocyte transplantation in the form of alginate microbead infusion. Microbeads are formed of hepatocytes encapsulated in sterile-alginate (*Novamatrix*) using an Encapsulator B-390 (*Buchi*) forming microbeads of approximately 500µM in size. These microbeads are infused into the intraperitoneal cavity via a wide bore cannula under ultrasound guidance. No immunosuppression is required as alginate is bio-inert and shields the hepatocyte from the immune system. Antibiotic prophylaxis as before is given. After full recovery (usually one month), or at time of liver transplant, the microbeads are removed using a laparoscopic peritoneal lavage.

Statistical Analysis

Data were analysed using GraphPad Prism V5 (*GraphPad Software La Jolla, CA*). Normality was tested for using the Kolmogorov-Smirnov normality test. For normally distributed data (MTT, viability at isolation, viability post-thawing and yield), data were evaluated using a two-tailed unpaired t-test. For non-parametric data (SRB, albumin and urea), data were analysed using the Mann-Whitney U test. In Chandler loop samples and clinical data, sample size was not sufficient for appropriate statistical tests. All data are expressed as mean \pm SEM. All data were analysed using GraphPad Prism V5 (*GraphPad Software La Jolla, CA*).

Results

Viability and Yield

Hepatocytes were isolated from 14 neonatal livers (Table 1; Figure 1; 3 DNDD, 11 DCDD), with an average viability of $89.4 \pm 1.8\%$ and average yield of $9.3 \times 10^6 \pm 2.0 \times 10^6$ cells/g. For comparison, hepatocytes were isolated from 14 adult livers (aged >16 years), with an average viability of $78.6 \pm 2.4\%$ and average yield $2.2 \times 10^6 \pm 0.5 \times 10^5$ cells/g (Figure 1A and 1B). Adult hepatocytes used as controls were taken from the latest 14 isolations in order to accurately represent the quality of cells that result from livers currently offered to the programme and to avoid era bias. Following cryopreservation and thawing, mean viability of neonatal hepatocytes was significantly higher compared to adult hepatocytes (Figure 1C, $77.6 \pm 3.6\%$ vs $59.2 \pm 2.5\%$ N=9, $P < 0.001$). MTT assay after cell plating demonstrated neonatal hepatocytes had significantly higher viability than adult hepatocytes (Figure 1D; OD; 1.0 ± 0.2 vs 0.4 ± 0.1 , $P < 0.05$). A lactate dehydrogenase (LDH) assay was used to measure plasma membrane damage of cryopreserved hepatocytes. Adult hepatocytes had significantly higher LDH leakage following cryopreservation and thawing compared to neonatal hepatocytes (Figure 1E; $70.7 \pm 10.5\%$ vs $43.4 \pm 5.2\%$, $P < 0.05$).

Cell Morphology and Attachment Efficiency

An SRB attachment assay showed cryopreserved neonatal hepatocytes had significantly higher attachment efficiency than adult hepatocytes (Figures 1F-1G: O.D, 2.4 ± 0.3 vs 0.6 ± 0.03 , $P < 0.01$). Images demonstrate neonatal hepatocytes formed complete monolayers on collagen-coated plates 24 hours post-plating, whereas adult hepatocytes are rounded with low confluence.

Hepatocyte Function

There was no significant difference in the production of liver specific proteins; albumin, alpha-1 antitrypsin and factor VII synthesis between neonatal and adult hepatocytes at 1-day post plating (Figure 2A-C; Albumin - 95.3 ± 33 vs 59.5 ± 18.1 ng/ μ g of cell protein/24h, N=8, $P > 0.05$, AAT – 122.5 ± 17.1 vs 91.1 ± 15.3 ng/ μ g of cell protein/24h, N=6, $P > 0.05$ and factor VII 3.0 ± 0.65 vs 6.78 ± 3.4 ng/ μ g of cell protein/24h, N=3 $P > 0.05$). Ammonia metabolism was comparable between neonatal

and adult cells as assessed by a ureagenesis assay (Figure 2D; 1.1 ± 0.3 $\mu\text{g/hr}/\mu\text{g}$ of protein vs 0.6 ± 0.4 $\mu\text{g/hr}/\mu\text{g}$ of protein, $N=5$, $P>0.05$). Phase II enzyme activity of neonatal and adult hepatocytes was compared by measuring resorufin conjugation. Neonatal hepatocytes had greater resorufin conjugation than adult hepatocytes, although this was not significant (Figure 2E; 531.8 ± 223.5 pg/hr vs 97.4 ± 51.0 pg/hr, $N=3$, $P>0.05$). Phase II enzyme function was further assessed by measuring activity of UDP glucuronosyltransferase (UGT) enzymes. Incubation with uridine 5'diphosphoglucuronic acid (UDPGA) and proluciferin UGT multi-enzyme substrates showed neonatal function of UGT enzymes was comparable to adult hepatocytes (Figure 2F; 30.0 ± 7.2 vs 37.7 ± 4.0 % UGT substrate consumed, $N=3$, $P>0.05$).

Drug Metabolism of neonatal hepatocytes

To determine the ability of neonatal hepatocytes to metabolize drug compounds, different cytochrome p450 isoforms were investigated following a 3-day induction. 3 out of 5 neonatal batches tested showed increased cytochrome p450 activity following induction with omeprazole (CYP1A2) and phenobarbital (CYP2C9 and CYP2B6) (Figure 3; Table 2 see supplementary information). Four out of five batches of neonatal hepatocytes showed increased activity of CYP3A4 and CYP3A7 following induction with rifampicin (Figure 3, Table 2 see supplementary information). These results are comparable to adult hepatocytes, which are not all inducible following the isolation and cryopreservation process (data not shown).

Phase I activity was further investigated by measuring the conversion of 7-ethoxyresorufin to resorufin. CYP1A1/1A2 of neonatal hepatocytes was comparable to that of adult hepatocytes following induction with omeprazole for 3 days (Figure 2; 1.9 ± 0.1 vs 3.3 ± 0.3 of resorufin formed/minute/million cells).

Neonatal hepatocytes and effect on thrombus formation *in vitro*

An *in vitro* tubing loop model (Chandler loop) was used to compare how neonatal and adult hepatocytes trigger activation of the coagulation cascade when in contact with ABO-matched blood (13,14). Adult hepatocytes elicited a visible thrombus formation and a drop in platelet count after 60

minutes compared to neonatal hepatocytes and control blood only samples (no clot formation) (Figure 4a; 28.3×10^9 (AH) vs 118.7×10^9 (NH) vs 214.3×10^9 cell/L, N=3). Furthermore, after 60 minutes adult hepatocytes caused a decrease in circulating white cells compared to neonatal cells (Figure 4b; 3.34×10^9 vs 6.63×10^9 vs 5.8×10^9 , N=3). There was no difference between neonatal and adult hepatocytes in the expression of the complement protein C5b-9. These preliminary results suggest neonatal hepatocytes may not trigger coagulation activation and platelet/white cell consumption to the same extent as adult hepatocytes. To investigate the potential mechanisms of IBMIR activation by neonatal hepatocytes, tissue factor expression was quantified. Immunofluorescent staining showed adult hepatocytes had strong expression of tissue factor in the cytosol. Tissue factor expression was absent in hepatocytes derived from neonatal hepatocytes (Figure 4d). This was further confirmed by qPCR analysis for the tissue factor gene. This showed that adult hepatocytes had significantly higher tissue factor expression than neonatal hepatocytes (Figure 4e, relative expression 2.06 ± 0.16 vs 0.32 ± 0.14 , N=5, $P < 0.001$). Cytokine analysis showed there were no differences in IL-2, IL-6, IL-8, VEGF, TNF- α , IL-1 α , MCP-1, EGF and IL1-RA, although neonatal hepatocytes did show a trend towards higher IL-10 production (see supplementary information)

Clinical use of Neonatal Hepatocytes

Three children have undergone transplantation of neonatal hepatocytes in our center to date. Two of these children underwent the procedure for ALF and received cryopreserved neonatal hepatocytes encapsulated in alginate microbeads injected into the intraperitoneal cavity while awaiting liver transplantation. One child with a rare inborn error of sulphite metabolism received cryopreserved neonatal hepatocytes via the intraportal route.

Child 1 and 2 with acute liver failure were aged 24 and 18 months respectively at time of hepatocyte transplantation. Child 1 was born prematurely and diagnosed with ALF due to neonatal hemochromatosis liver disease (siderosis on salivary gland biopsy) with persistent and worsening liver function despite treatment with exchange transfusion and intravenous immunoglobulin. She was listed for liver transplantation but due to her small size an organ was not readily available. The patient underwent hepatocyte microbead transplantation 12 days after admission; (40ml of infusate

containing 3×10^7 /kg neonatal cells (first infusion) and 2.25×10^7 /kg neonatal cells (second infusion) which was tolerated well without any adverse effects. This patient survived for a further 31 days with supportive management at which time a suitable organ became available and she underwent LT. Microbeads were washed out at time of the LT. Adhesions were not noted at laparotomy. She is well now 2.5 years post-transplant.

Child 2 presented with coagulopathy (INR of 7) and encephalopathy at the age of 18 months. He was listed for liver transplantation but as no organ became immediately available he underwent microbead transplantation as a bridging intervention day 2 of admission. The patient received a total of 1.7×10^7 /kg neonatal hepatocytes and made a full recovery with a normal INR 10 days post microbead transplantation and he did not suffer any adverse events. Microbeads were washed out 6 months post infusion at which time some fibrous bands between clumps of cells and the omentum were described at laparoscopy. Nine months later he presented with an INR of 17 and encephalopathy at which time he was listed once again for LT and received an organ shortly after listing. At time of LT, clumps of microbeads were retrieved but adhesions were not observed and the child remains well 6 months post liver transplantation.

Child 3 was diagnosed with a rare inborn error of sulphite metabolism, which is associated with irreversible neurological damage and early death. The patient underwent intraportal infusion of a total 6.6×10^8 cryopreserved hepatocytes over 4 infusion, with the first transplantation on day 2 of life. Biochemical stability was achieved, with sulphite and urinary s-sulfocysteine lower than expected, given previous experience with infants suffering this condition, though it is not possible to draw firm conclusions regarding the efficacy of hepatocyte transplantation from the improvement in biochemical parameter alone.

Discussion

We suggest that neonatal livers, currently not used for organ transplant may be an ideal cell source for hepatocyte transplantation. Neonatal hepatocytes have high viability, attachment efficiency and metabolic function post-cryopreservation and may be a suitable alternative to adult hepatocytes. Results from the Chandler loop model suggest neonatal hepatocytes may not activate coagulation to the same extent as adult hepatocytes and may express less TF. In a small sample of patients, neonatal hepatocytes were shown to be suitable for clinical hepatocyte transplantation, with no patients showing adverse effects.

The ability to cryopreserve hepatocytes is essential so they can be stored until needed for emergency treatment. This is one of the major advantages of hepatocyte transplantation versus LT. However, the cryopreservation process adversely affects viability, function and attachment efficiency of hepatocytes, with LDH leakage significantly increased upon thawing (15). Hepatocytes derived from neonatal hepatocytes are able to withstand the cryopreservation and thawing process significantly better than adult hepatocytes, with significantly greater viability and less LDH leakage. Further research is required into the molecular membrane structure of neonatal and adult hepatocytes and how this may affect cryopreservation. The use of neonatal hepatocytes that have high viability post thawing has the potential to increase the stock of good quality hepatocytes used for off-the-shelf emergency hepatocyte transplantation.

Fully functional neonatal hepatocytes are vital to the success of hepatocyte transplantation to provide sufficient enzyme function to rescue the enzyme deficient recipient. However, theoretical concerns have arisen about the functional maturity of neonatal cells. Newborn liver function is still in development during the early post-natal period and there is a decreased ability of the neonatal liver to metabolize and detoxify xenobiotic substances (16). We have shown albumin, alpha-1 antitrypsin, factor VII synthesis and ammonia metabolism in neonatal hepatocytes is comparable to adult hepatocytes, suggesting neonatal hepatocytes are functionally suitable for transplantation. Studies into the development of hepatic drug metabolizing enzymes have found that each enzyme system has its

own pattern of development (17). CYP3A7 is the primary isoform expressed in the neonatal period and declines rapidly after birth, with CYP3A4 taking over its function within the first few weeks of life. We found 4 out of 5 of the neonatal cells tested still had inducible CYP3A7 and CYP3A4 activity. The expression of CYP1A2 is the last enzyme to develop, only present by 1 to 2 months of life (17). This is reflected by our results, with very low levels of CYP1A2 induction in 3 out of 5 neonatal cells. Higher levels of induction in some neonatal cells may be related to drugs received prior or shortly after birth, including phenobarbital and midazolam. Quantitative data into the development of the phase II enzymes is limited but it has been suggested that the UGT enzymes, which metabolize 15% of all drugs, are triggered at birth and reach adult levels by 3 months (17). This suggests neonatal hepatocytes should have functioning phase II enzymes which is reflected in our results that show resorufin conjugation and UGT substrate consumption was similar to adult cells.

In animal models, up to a 6-fold loss of hepatocytes has been observed within the first 24 hours of cell transplantation, with studies relating this early cell loss to activation of coagulation and complement cascades described as the instant blood mediated inflammatory reaction (IBMIR) (13,18–20). The IBMIR is triggered by the expression of tissue factor on the donor hepatocytes (21). Neonatal cells may potentially be less thrombotic and immunogenic than adult liver derived cells allowing increased engraftment and function. Initial results using the Chandler loop model suggest neonatal hepatocytes do not cause platelet consumption and circulating white cell loss to the same extent as adult hepatocyte. There is some variability in the platelet counts of blood samples containing neonatal hepatocytes compared to adult hepatocytes, which may be related to the age of the donor. One of the donors used in this experiment was aged 4 days whereas the other two were aged 6 weeks and 7 weeks respectively. Further experiments are required to determine if lower immunogenicity is observed within the first few days of life. Significant differences have been described between neonatal and adult coagulation systems with concentrations of vitamin K dependent factors II, VII, IX and X and contact factors XI and XII reduced to about 50% of normal adult values (22–24). Furthermore, activated partial thromboplastin time is prolonged in healthy full-term infants compared to adults (25). We found tissue factor expression was decreased in neonatal hepatocytes compared to

adult cells, which may partly explain the decreased coagulation activation. Potentially, if neonatal cells do not activate coagulation to the same extent as adult hepatocytes, cells have a much better chance of cell survival, providing sustained function for children with metabolic liver disease, without the need for multiple infusions.

Previous work published on neonatal hepatocytes considered warm and cold ischaemic times a major limiting factor in isolating good quality hepatocytes, with ideal cold ischaemic time being limited to 2.5 hours and warm ischemia time not exceeding 30 minutes, based on previous *in vitro* studies (26–28). However, we have shown cold ischemia times of up to 8 hours and warm ischemia times of up to 84 minutes can still produce hepatocytes with a high viability and yield. It is possible neonatal organs are able to withstand longer periods of ischemia due to their high quality yet the mechanisms involved in this process remain to be found.

Although we have characterized neonatal and adult hepatocyte viability and function *in vitro*, we have yet to prove their clinical efficacy. A larger patient cohort is required to validate the enzymatic function of these cells in a range of liver-based metabolic diseases.

Conclusions

The use of neonatal livers as a cell source for hepatocyte transplantation may significantly increase the number of high quality cells available for transplantation, with the advantage of excellent cell viability, function and potentially lower activation of the IBMIR of cells. Neonatal hepatocytes have been used in a clinical context and though proof of clinical efficacy is still elusive, non-invasive markers of immune activation were decreased with use of neonatal cells. This cell source may significantly improve the clinical outcomes of hepatocyte transplantation for paediatric ALF or in-born errors of metabolism.

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Figure Legends

Tables

Table 1. Donor details from neonatal livers used to isolate primary hepatocytes. *born at 23 weeks + 5 days. DNDD=donation after neurological determination of death

Figures

Figure 1 Viability, yield and attachment efficiency of human hepatocytes isolated from neonatal and adult livers (A) Cell viability upon hepatocyte isolation, Neonatal N=14, Adult N=14 (B) Cell yield per gram of liver tissue, Neonatal N=14, Adult N=14 (C) Cell viability following cryopreservation and thawing, Neonatal N=9, Adult N=9 (D) Viability assessed by MTT assay, Neonatal N=9, Adult N=9 (E) Plasma membrane damage assessed via lactate dehydrogenase (LDH) release, N=5 (F) Cell attachment assessed by SRB assay, Neonatal N=6, Adult=6 (G) Cell morphology of neonatal (left) and adult hepatocytes (right) 24 hrs post plating on collagen coated plates *P<0.05, **<0.001, ***P<0.001. Data represents mean±SEM

Figure 2 Metabolic functions of neonatal and adult human hepatocytes (A) Albumin synthesis measured by ELISA N=8 (B) Alpha-1 antitrypsin synthesis measured by ELISA N=6 (C) Factor VII synthesis measured by ELISA N=3 (D) Ureagenesis measured using a quantichrome urea assay following incubation with 5mM ammonium chloride N=5 (E) Phase II activity measured by the metabolism of resorufin N=3 (F) Activity of UGT was measured by glucuronidated luciferin production following incubation with 50µM uridine 5'diphosphoglucuronic acid (UDPGA) and proluciferin substrates (50µM UGT multienzyme mix) N=3 N.S = not significant, P>0.05. Data represents mean±SEM.

Figure 3 Induction of cytochrome p450 enzymes in human neonatal and adult hepatocytes.

Hepatocytes were cultured for 24h at 37°C, 5% CO₂ and cytochrome metabolism induced every day for 3 days before detection of cytochrome activity (A) CYP1A2 activity in neonatal hepatocytes measured by Luciferin-ME following treatment with 50µM omeprazole (OMP) or DMSO control (B) CYP2C9 activity in neonatal hepatocytes measured by Luciferin-H following treatment with 1mM phenobarbital (PHE) or DMSO control (C) CYP2B6 activity in neonatal hepatocytes measured by Luciferin-2B6 following treatment with 1mM phenobarbital (PHE) or DMSO control (D) CYP3A4 activity in neonatal hepatocytes measured by Luciferin-IPA following treatment with 25µM rifampicin (RIF) or DMSO control (E) CYP3A7 activity in neonatal hepatocytes measured by Luciferin-PFBE following treatment with 25µM rifampicin (RIF) or DMSO control. (F) Cytochrome P450-1A1/1A2 activity in neonatal and adult hepatocytes was assessed by measuring the conversion of 7-ethoxyresorufin to resorufin following induction with 50µM omeprazole or DMSO controls for 3 days.

Figure 4 Coagulation and complement activity in ABO-matched blood following incubation with neonatal and adult human hepatocytes in the Chandler loop model (A) Platelet count (B) White cell count (C) C5b-9 expression N=3 (D) Chandler loop design PVC tubing (3/16"x1x16"x40cm) was filled with 6mls ABO matched blood and hepatocytes. Loops were closed into circuits with heparin coated 3/16" polystyrene connectors. Tubing loops were rotated at 24rpm and incubated at 37°C for 0, 15, 30 and 60 minutes. (E) RNA was extracted from neonatal and adult hepatocyte lysates. qPCR analysis for the tissue factor gene was carried out using Applied Biosystems QuantStudio 7 Flex Real Time PCR system (*Thermo Fisher Scientific, Paisley, UK*). Results are normalised to expression of the housekeeping gene β -actin. N=5, ***P<0.001. (F) Tissue Factor expression in hepatocytes isolated from adult and neonatal livers. Cryopreserved hepatocytes were thawed, plated in chamber slides overnight at 37°C, 5% CO₂ and stained with a rabbit monoclonal tissue factor antibody. (A) Tissue factor expression (B) DAPI.

